A Two-Holed Story: Structural Secrets About ClC Proteins Become Unraveled?

ClC Cl- channels are found in almost all organisms, ranging from bacteria to mammals, in which nine Cl- channels belonging to the ClC family have been identified. The biophysical properties and physiological functions of ClC Cl- channels have been extensively reviewed. In this short review, we will focus on recent results obtained on the X-ray structure and functional properties of the prokaryotic ClC-ec1 protein and some results obtained on the role of the cytoplasmic COOH terminus of mammalian ClCs.

CIC channels can be classified into plasma membrane channels and organelle channels. ClCs that function in the plasma membrane (ClC-1, ClC-2, ClC-Ka, and ClC-Kb (in mammals)) are involved in the stabilization of membrane potential and in transepithelial transport. The presumed function of most intracellular organelles (ClC-3, ClC-4, ClC-5, ClC-6, and ClC-7 (in mammals)) is support of the acidification of the intraorganellar compartment. The association of mutations in several ClC genes with various human diseases confirms the relevance of their functions (17, 21).

Knockout studies have greatly helped in elucidating the physiological function of several ClCs, even though for some of them their precise role is not clear (4, 5, 22, 33, 44, 48). Model organisms have been very helpful for understanding several aspects of ClC proteins. The nematode Caenorhabditis elegans provides a good system to define the physiological functions of ClC proteins. In fact, this organism owns six ClC genes representative of the mammalian ClCs (45).

Bacterial ClCs have allowed the first determination of the X-ray structure of a ClC homolog (9). Furthermore, recent experiments on reconstituted bacterial ClCs revealed an unexpected function as a Cl-/H+ antiporter (2). A unique feature of ClC channels is their homodimeric architecture in which each subunit forms a proper pore. Such a structure had been conjectured early on by Chris Miller, who studied Torpedo ClC-0 channels reconstituted in planar lipid bilayers (28). Measuring single channels, Miller observed that at negative potentials the channel fluctuated between three different conductance levels, one closed and two open states. The open events occurred in bursts separated by long closures. The substates visible during a burst had an identical conductance of ~8 pS, and the respective open probabilities were distributed binomially as if created by two independent channels. The long closure events demonstrated, however, the presence of a "common" gate that acts on both "protopores" simultaneously and that exists in parallel to the individual protopore gates (FIGURE 1). This "double-barreled shotgun" model (28) was later con-
firmed by using mutation analysis. It was shown that the protopore gate, the conductance, and the ion selectivity are determined by the amino acid sequence of a single subunit; on the other hand the common gate depends on the properties of both pores of CIC-0 (24, 27). Qualitatively, a similar double-barreled appearance was shown also for CIC-1 (37) and CIC-2 (49). Based on the recent structure determination of bacterial CICs (9) it can be assumed, even though not demonstrated, that a double-barreled architecture holds true for all CIC proteins.

Most CIC Cl– channels that have been studied are characterized to be relatively selective for Cl– over other anions (the selectivity sequence generally is Cl– > Br– > I–; see Ref. 35 for review). Another general feature is their voltage-dependent gating that seems to be tightly coupled to the permeation of Cl– ions. In addition to the strong dependence on intra- and extracellular Cl– concentration, these channels are regulated by intra- and extracellular pH (35).

The organelle channels CIC-3, CIC-4, and CIC-5 can be classified as a subfamily characterized by a strong outward rectification when expressed in Xenopus oocytes or mammalian cells (16, 23, 43). CIC-6 and CIC-7 (6) form the third branch of the CIC family. So far they cannot be expressed functionally as Cl– channels in heterologous systems (21).

Structure of CIC proteins

The double-barreled architecture of CIC proteins has been fully confirmed by solving the X-ray structure of two prokaryotic CIC homologs [one from Salmonella typhimurium and one from Escherichia coli (9)]. The bacterial proteins became of great interest after the first crystallization of a bacterial K+ channel (8). Prokaryotic CIC proteins are significantly homologous to their eukaryotic counterparts. In particular, they have the same transmembrane topology and display several highly conserved stretches that were later to be shown to form Cl– ion-binding sites in the center of each subunit (10, 30).

The first solved X-ray structure of the S. typhimurium ClC protein (StClC) has been determined at 3.0-Å resolution (9). The protein is formed by two identical subunits that each have a roughly triangular shape when viewed from top or from bottom. They are related to each other by an almost perfect symmetry of a 180° rotation and an extensive interaction surface that is perpendicular to the plane of the membrane. Each subunit contains an amazingly complex fold of 18 α-helices that reveals an internal antiparallel pseudosymmetry: the NH2-terminal half of each subunit has the same fold as the COOH-terminal half and also a spurious sequence homology to it but is inserted into the membrane in the reverse direction, forming a “sandwich,” similar to what has been seen for aquaporin water channels (46). More recently, by employing co-crystallization with Fab fragments, Dutzler et al. (10) succeeded in obtaining an improved 2.5-Å resolution structure of the E. coli protein ClC-ec1. Further discussion of the struc-

![FIGURE 2. Structure of CIC-ec1](image-url)
The most important feature of the structure is that in the center of each subunit two nearby Cl– ions are clearly resolved, indicating the presence of at least two anion-binding sites, called S

\( ^{\text{in}} \) and S

\( ^{\text{ext}} \) for the more intracellularly and the more centrally located site, respectively (Figure 3). The protein regions that contribute to the formation of the sites are the ones that are most conserved among all ClC proteins, suggesting clearly that they form the selectivity filter of the conduction pathway. The fact that the binding sites of the two subunits are far from each other (~40 Å) is fully compatible with the independence of the two protopores of the double-barreled shotgun model. The central Cl– ion is completely buried in the protein and stabilized by the positive dipole moments of helices F and N that point their NH\(_2\) terminus toward the ion and by the interaction with several backbone amide hydrogens and the OH groups of serine 107 and tyrosine 445. The more intracellularly localized Cl– ion appears to be in direct contact with the intracellular solution (Figure 3). Although these results identify the selectivity filter, the permeation pathway is not evident from the structure. In particular, a negatively charged glutamate residue (E148) appears to block the movement of the centrally bound Cl– ion toward the extracellular solution. Also, serine 107 seems to be a major obstacle for Cl– permeation toward the intracellular solution (29). It has thus been proposed that the crystallized structure corresponds to a closed channel and that the glutamate side chain may act as a gate that obstructs the pore from the extracellular side.

Two different approaches have been used to study in more detail the function of this glutamate. First, the X-ray structure of mutant E. coli proteins, in which the glutamate has been replaced by alanine and glutamine (E148A, E148Q), has been determined (10). In both mutant structures a third Cl– ion was found at the position occupied by the E148 side chain in the wild-type (WT) structure. In the E148Q structure the glutamine side chain is oriented in the extracellular direction, suggesting that in the conducting conformation of the WT the glutamate side chain could be similarly orientated, allowing the permeation of Cl– ions. Otherwise, the mutant structures are virtually identical to the WT structure.

Because at the time of these structural studies the bacterial protein could not be measured by using electrophysiological methods, the Torpedo ClC-0 was used as a model to explore the functional effects of E148 mutations (E166 in ClC-0). All three mutations studied (E166A, E166V, E166Q) almost completely abolished the closure of the protopore gate, resulting in permanently open channels (10). In addition, WT ClC-0 can be converted to a similarly permanently open channel by a reduction of the extracellular pH (10), as if protons open the channel by protonating the glutamate side chain. Together, these results have led to the proposal that the gating of ClC channels is regulated by a simple mechanism in which the glutamate side chain acts as a gate occluding the ion pathway. Although several details may not be easily explained (3, 47), this simple model is qualitatively in accordance with several properties of ClC-0 gating. First, it is consistent with the independence of the two pores because each pore has its own glutamate. The coupling between Cl– ion conduction and gating (36) could be explained by a competition between E148 and Cl– ions, and the pH effects on gating (7, 10) are caused by a direct protonation of E148.

The ClC-ec1 from E. coli is Not a Channel

Little is known about the ClC functions in prokaryotic organisms. Recently, two E. coli ClC genes (ClC-ec1 and ClC-ec2) have been proposed to be involved in the extreme acid resistance response that permits enteric bacteria to survive in the stomach by providing an electrical shunt that helps in the bacterial acid-extrusion mechanisms (20).
In the past, since all attempts to measure electrical currents of bacterial ClCs reconstituted in lipid bilayer failed, ClC-ec1 function could be assayed only by measuring Cl− fluxes in reconstituted liposomes (20, 25). From these flux studies, Maduke and co-workers (25) proposed that ClC-ec1 functions as a channel displaying a high selectivity for anions over cations and a selectivity sequence similar to that found for CIC-0 and CIC-1.

Recently, Accardi et al. (1) succeeded in obtaining an extremely pure and high-yield preparation of ClC-ec1 protein that allowed an electrophysiological characterization in planar lipid bilayers. Only macroscopic currents could be measured, and from the noise properties a very small single-channel conductance (<0.1 pS) was inferred (1). The current did not show the characteristic voltage and time-dependent gating of the eukaryotic CIC channels, exhibiting a practically linear current-voltage (I−V) curve. Importantly, ClC-ec1-carried currents were activated by low pH, confirming earlier results from Cl− flux measurements (20). To test the proposed role of E148 in proton sensing, Accardi and coworkers (1) studied the mutant E148A. They found that the mutation completely abolishes the pH dependence, in agreement with the expectations from the structural data on ClC-ec1 and the functional data on CIC-0.

In all previous studies, it has implicitly been assumed that the bacterial ClC-ec1 is a Cl− channel just like CIC-0. This assumption was supported by ion selectivity sequence inferred from flux measurements (24) and by studies that successfully used the structure of the bacterial protein as a guide for mutagenesis to identify residues involved in the binding of two organic inhibitors of ClC-1 [9-anthracencarboxylic acid (9-AC) and phenyl chlorophenoxyacetic acid (CPA)] (13). Thus it came as a big surprise when Accardi and Miller discovered that the bacterial protein is not an ion channel but rather behaves as a H+/Cl− exchange transporter (2). The first indication was that the measured reversal potential in a Cl− gradient was significantly smaller than that expected by Nernst’s equation, suggesting the presence of another permeating ion, indicating that H+ could be the second ion passing through ClC-ec1, but they could not prove this directly with the stoichiometric ratio of ~2, indicating that two Cl− ions are transported for each proton. In principle, it cannot be strictly ruled out that the true stoichiometric ratio is somewhat smaller than 2 and that some Cl− slippage occurs. However, the very good fit of the data by Eq. 3 argues against this objection that would predict a deviation from Eq. 3.

These results led to the confirmation that H+ is the second ion passing through ClC-ec1, but they suggest that the prokaryotic ClC-ec1 acts as an H+/Cl− exchange transporter and not as a channel (2). The most crucial experiments substantiated this hypothesis: flux measurements in liposomes showed that ClC-ec1 is able to transport Cl− against its electrochemical gradient in the presence of a proton gradient and vice versa (2). Together these experiments show that ClC-ec1 operates as a Cl−/H+ antipporter with an apparent stoichiometric ratio of 2 Cl−:1 H+. Interestingly, the mutation E148A of the crucial glutamate residue led to a complete loss of transporter activity and showed a purely electrodiffusive Cl− transport (2), indicating that E148 is centrally involved in the H+ transport. ClC-ec1 is significantly homologous to the eukaryotic CIC proteins, but of course the transporter function of ClC-ec1 does not imply that all CICs are necessarily transporters. CIC-0, CIC-1, CIC-2, and CIC-K are clearly Cl− channels. However, their gating is strongly dependent on pH, indicating a kind of memory of the bacterial transporter. This notion is also supported by the conserved crucial role of E148 for CIC gating in all CIC channels studied (10, 13, 14, 16, 32, 40, 47). The new findings could be also helpful in future studies on some human channels, such as CIC-6 and CIC-7, that are still poorly understood.

Role of the Cytoplasmic CBS Domains

The crystallized prokaryotic CICs have only short intracellular NH2 and COOH terminals, whereas these terminals are often long in the eukaryotic channels, where they play important roles in channel function (11).
Initial experiments have shown that the COOH terminus of ClC-0 is involved in the common gate and is important for the functional expression of ClC channels (15, 26). Interestingly, Maduke et al. (26) showed that functional channels could be obtained by the injection of two separate constructs encoding the NH₂-terminal part comprising all intramembraneous segments and the cytoplasmic COOH terminus, respectively. Later it was found that the COOH terminus of eukaryotic ClC channels contains two so-called cystathionine-β-synthase (CBS) domains (34). Several different types of proteins contain CBS domains, most often in pairs. Crystal structures of the enzyme inosine monophosphate dehydrogenase demonstrate that the CBS domains are folded into two amphipatic α-helices and three β-strands and revealed that the two CBS domains interact with each other through their β-strands, whereas the α-helices are oriented away from the interaction site (42, 50). Their function is still unknown, but several mutations occurring in CBS domains are found in hereditary human diseases involving ClC channels, such as congenital myotonia, hypercalciuric nephrolithiasis, osteopetrosis, Dent’s disease, and Bartter’s syndrome, suggesting that they play a crucial role in the physiological functions of these channels (see Ref. 12 for references).

Recently, it has been shown that the CBS domains from several proteins including ClC-2 form a binding site for adenosine derivatives such as AMP and that disease-causing mutations influence the binding of these adenosine derivatives (41). This finding led to the interesting hypothesis that CBS domains act as sensors of the cellular energy status.

Two recent studies employed different techniques to explore in more detail the function of CBS domains in ClC channels, in particular in ClC-1 (12, 18). Previously, it had been shown that truncating ClC-1 after CBS1 does not give rise to any current (39). In the latest work, Estévez and co-workers (12) used co-immunoprecipitation, electrophysiology, and surface-expression measurements using an extracellular epitope to show that both CBS domains are needed for the rescue of function and localization of the separately expressed NH₂-terminal half of the channel. Moreover, the replacement of CBS domains of ClC-1 with the CBS domains of other ClC channels preserved function, showing that the structure and function of CBS domains from ClC channels are highly conserved (12). Results obtained by co-expression of different constructs indicated that the CBS domains interact (FIGURE 4A). An important observation of this study was that some residues located in CBS2 are involved in the voltage dependence of gating by affecting or abolishing the common gate of ClC-0 and ClC-1. This is a confirmation of previous results showing that parts of the COOH terminus of ClC-0 are involved in the common gate of ClC-0 (15). Interestingly, the in-frame deletion of CBS2 but not of CBS1 was found to be tolerated in ClC-1, demonstrating that CBS2 is not strictly essential for function (FIGURE 4A). Hebeisen and co-workers (18) have studied the role of CBS domains of ClC-1 using a different approach and...
obtaining discordant results. First, measuring the surface expression of truncated green fluorescent protein-bosed CIC-1 by confocal microscopy, they found that CBS domains are not necessary for the insertion into the plasma membrane. Second, to test whether the two domains interact they coexpressed concatameric constructs containing the two main parts of the dimeric protein with smaller constructs containing various COOH-terminal cytoplasmic pieces. From these experiments it appeared that the CBS domains do not bind each other and that there are no interactions between the COOH terminals of the two subunits (FIGURE 4B). Moreover, they observed that mutations in the COOH terminus did not alter the gating properties of the channel but cause a decrease in the maximal current. Surprisingly, Hebeisen et al. (18) found that the deletion of CBS1 but not that of CBS2 was tolerated, in complete contrast to the results of Estévez et al. (12) (FIGURE 4). These two studies underline the difficulties involved in investigating the role of CBS domains. The use of different methods and constructs used in these two studies (12, 18) is probably one of the reasons for such discordant results. For example, the employment of concatameric construct could force an assembly and a targeting different from that found in coexpression studies. The divergent conclusion regarding the necessity of CBS1 remains unclear. Regarding the necessity of CBS2, the deletion construct used by Estévez et al. retained the last 117 amino acids, whereas Hebeisen et al. used a truncation before CBS2. Thus it can be concluded that the terminal amino acids after CBS2 have an important functional role in CIC-1. Caution must be applied to all of these interpretations, however, because divergent results are also obtained if other CIC channels (e.g., CIC-0) are used for the CBS deletions (12).

An apparently fundamentally different conclusion was reached by Hebeisen et al. and Estévez et al. regarding the relationship of the CBS domains with the double-barreled architecture: Hebeisen et al. concluded that the CBS domains of one subunit support the function of and interact only with the subunit to which they belong and not with the neighboring subunit, whereas Estévez et al. found that mutations in only one CBS2 domain abolished or altered the common gate in a dominant fashion in heterodimeric mutant WT CIC-0 channels. The conclusion of Hebeisen et al. was mainly based on the use of concatameric constructs in which one of the subunits bore a point mutation (SS37F) that renders the channel less sensitive to block by 9-AC (13). The authors assumed that 9-AC block represents a property of a single pore not influenced by the neighboring subunit (18). However, this assumption is probably not true, because results with CIC-0 indicate a dependence of 9-AC and CPA block on the common gate (31) (Pusch, unpublished result). In conclusion, it seems fair to say that we still have a lot to learn about the role of the intracellular domains in CIC channels.

Outlook

The past several years have seen great steps forward in the understanding of the structural and functional properties of CIC Cl- channels, or better to say now CIC Cl-–transporting proteins. The X-ray structure of bacterial CIC proteins marked a breakthrough in the CIC field. However, we have also learned an important lesson: the three-dimensional atomic structure of a protein alone does not provide enough information to understand its function. The structure of the bacterial CIC-ec1 by no means revealed that it is a transporter that shuffles Cl- ions in one direction strictly coupled to the proton transport in the opposite direction. Only functional electrophysiological analysis revealed this unexpected behavior, demonstrating the importance of using various approaches to unravel the secrets of biology. Further analysis is clearly needed to define the mechanism of the countertransport of Cl- and H+ in CIC-ec1 and also to investigate the relation between the bacterial transporter and the pH dependence of eukaryotic CIC Cl- channels.

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